

## Properties of substance P-stimulated mucus secretion from porcine tracheal submucosal glands

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**Khansaheb M, Choi JY, Joo NS, Yang Y, Krouse M, Wine JJ.** Properties of substance P-stimulated mucus secretion from porcine tracheal submucosal glands. *Am J Physiol Lung Cell Mol Physiol* 300: L370–L379, 2011. First published December 3, 2010; doi:10.1152/ajplung.00372.2010.—Human and pig airway submucosal glands secrete mucus in response to substance P (SubP), but in pig tracheal glands the response to SubP is >10-fold greater than in humans and shares features with cholinergically produced secretion. CFTR-deficient pigs provide a model for human cystic fibrosis (CF), and in newborn CF pigs the response of tracheal glands to SubP is significantly reduced (Joo et al. *J Clin Invest* 120: 3161–3166, 2010). To further define features of SubP-mediated gland secretion, we optically measured secretion rates from individual adult porcine glands in isolated tracheal tissues in response to mucosal capsaicin and serosal SubP. Mucosal capsaicin ( $EC_{50} = 19 \mu\text{M}$ ) stimulated low rates of secretion that were partially inhibited by tetrodotoxin and by inhibitors for muscarinic, VIP, and SubP receptors, suggesting reflex stimulation of secretion by multiple transmitters. Secretion in response to mucosal capsaicin was inhibited by CFTR<sub>inh</sub>-172, but not by niflumic acid. Serosal SubP ( $EC_{50} = 230 \text{ nM}$ ) stimulated 10-fold more secretion than mucosal capsaicin, with a  $V_{\text{max}}$  similar to that of carbachol. Secretion rates peaked within 5 min and then declined to a lower sustained rate. SubP-stimulated secretion was inhibited 75% by bumetanide, 53% by removal of  $\text{HCO}_3^-$ , and 85% by bumetanide + removal of  $\text{HCO}_3^-$ ; it was not inhibited by atropine but was inhibited by niflumic acid, clotrimazole, BAPTA-AM, nominally  $\text{Ca}^{2+}$ -free bath solution, and the adenylate cyclase inhibitor MDL-12330A. Ratiometric measurements of fura 2 fluorescence in dissociated gland cells showed that SubP and carbachol increased intracellular  $\text{Ca}^{2+}$  concentration by similar amounts. SubP produced rapid volume loss by serous and mucous cells, expansion of gland lumina, mucus flow, and exocytosis but little or no contraction of myoepithelial cells. These and prior results suggest that SubP stimulates pig gland secretion via CFTR- and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels.

cystic fibrosis; lung disease; neurotransmitter; C-fiber; tachykinins; ion channel

AIRWAY SUBMUCOSAL GLANDS EXPRESS CFTR (10) and participate in the innate defenses of the airways (6). Substance P (SubP) stimulates airway submucosal gland secretion in humans and pigs (5, 37). In humans, stimulation is weak and occurs mainly via a CFTR-dependent process (5); in pig tracheal glands, the response to SubP is >10-fold greater than in humans (5), and it shares features with cholinergically produced secretion (37). The porcine lung has many features in common with human lungs, suggesting that cystic fibrosis (CF) pigs could provide

good models of human CF lung disease (31). CFTR-targeted pigs (CFTR<sup>+/-</sup> and CFTR<sup>+/ $\Delta F508$</sup> ) have been produced (32) and bred to produce CF pigs that display early features of CF airway disease (16, 33, 36) and, subsequently, develop multiple features of CF airway disease, including bacterial infections and mucus accumulation (36, 39). Tracheal submucosal glands of newborn CF pigs secrete significantly less mucus in response to SubP than do newborn controls (16), indicating at least partial dependence on CFTR.

The large difference in the efficacy of SubP in mediating airway glandular secretion between pigs and humans may be relevant to the use of CF pigs as models of human CF. In the research reported here, we optically measured mucus secretion from single submucosal glands of adult pigs in an attempt to further delineate the mechanisms and functional significance of SubP-mediated secretion in pig airways. We used mucosal capsaicin to activate SubP-containing afferents and stimulate glands reflexly, as was done previously in mice (14) and humans (5), and serosal SubP to stimulate neurokinin (NK-1) receptors on gland cells directly. These approaches, which complement methods that measured fluid secretion in response to SubP in intact bronchi (1, 2, 25, 37), provide a basis for evaluating single-gland studies of newborn (16) and, eventually, adult CF pigs.

The results show that mucosal capsaicin stimulated CFTR-dependent secretion via multiple transmitters, while serosal SubP stimulated 10-fold more secretion, with a  $V_{\text{max}}$  and temporal response profile similar to carbachol. SubP-stimulated secretion was dependent on  $\text{Cl}^-$  and  $\text{HCO}_3^-$  and was inhibited by blockers of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  (CaCC) and  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  ( $\text{K}_{\text{Ca}}$ ) channels and by reductions of intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) and intracellular cAMP concentration. SubP produced rapid volume loss by serous and mucous cells, expansion of gland lumina, mucus flow, and exocytosis but little or no contraction of myoepithelial cells. These and prior results (16) suggest that SubP stimulates gland secretion via CFTR and CaCC channels.

### METHODS

For all except the fura 2 experiments, pig tracheas were obtained from fresh carcasses of 35- to 50-kg juvenile Yorkshire pigs of either sex after acute experiments carried out for other purposes; the experiments did not use any anticholinergics or other agents that might be expected to alter gland function. Pigs were cared for and euthanized according to protocols approved by the Institutional Animal Care and Use Committee at Stanford University. No pigs were killed specifically for these experiments. Approximately 55 pig tracheas were used for the gland secretion experiments, and 6 pigs were used for differential interference contrast (DIC) experiments. For fura 2 measurements of  $[\text{Ca}^{2+}]_i$ , three pigs were used; these experiments were

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approved by the Committee on Animal Research at Yonsei Medical Center, and all experiments with animals were performed under appropriate guidelines.

Pig tissues were transported to the laboratory in cold Physiosol solution (Abbott Laboratories) and then transferred to ice-cold Krebs-Ringer bicarbonate (KRB) buffer bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>, where they were maintained until use. The KRB buffer composition was 115 mM NaCl, 2.4 mM K<sub>2</sub>HPO<sub>4</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 1.2 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 10 mM glucose, and 1.0  $\mu$ M indomethacin (pH 7.4), and osmolality was adjusted to ~290 mosM. For HCO<sub>3</sub><sup>-</sup>-free experiments, 25 mM HCO<sub>3</sub><sup>-</sup> in the KRB buffer was replaced with 1 mM HEPES + 24 mM NaCl that had been pre-gassed with humidified 100% O<sub>2</sub>. This HEPES concentration maintained the bath pH at 7.4 after the solution was gassed with O<sub>2</sub>.

To prepare tissues for optical recording of secretion by individual glands, a ~0.5-cm<sup>2</sup> piece of ventral trachea or bronchus was pinned mucosal-side-up, and the mucosa with underlying glands was dissected from the cartilage and mounted in a 35-mm-diameter Sylgard (Dow Corning)-lined plastic petri dish, with the serosa in the bath (~1 ml volume) and the mucosa in air. This dissection removes the posterior membranous portion of the trachea, where local neurons are abundant, but additional local neurons are retained throughout the tracheal wall (40). The tissue surface was cleaned and blotted dry with cotton swabs and further dried with a stream of gas; then water-saturated mineral oil (20–30  $\mu$ l) was placed on the surface. The tissue was warmed to 37°C at a rate of ~1.5°C/min and continuously superfused with warmed, humidified 95% O<sub>2</sub>-5% CO<sub>2</sub>. For basolateral stimulation, pharmacological agents were diluted to final concentration with warmed, gassed bath solution and added to the serosal side by complete bath replacement. For apical stimulation with capsaicin, the compound at 10 $\times$  final concentration in oil was added to the premeasured oil layer and allowed to diffuse.

**Optical measurements.** Bubbles of mucus within the oil layer were visualized by oblique illumination, and digital images were captured with the macro lens of a Nikon digital camera or by mating a digital camera to one ocular of a Wild stereomicroscope. Each image contained an internal reference grid to compensate for any minor adjustments in magnification during the experiment. Stored images were analyzed by direct measurement or with ImageJ software (<http://rsb.info.nih.gov/ij/>). Mucus volumes were determined from the size of the spherical bubbles; bubbles that were not approximately spherical were omitted from secretion rate analyses. Details of these methods are described elsewhere (19).

**Estimating the proportion of responding glands.** In previous experiments, we found carbachol to be the most efficacious agonist for stimulation of mucus secretion. Therefore, we used responsiveness to a 5-min exposure to 10  $\mu$ M carbachol at the end of experiments to estimate the number of viable glands in each preparation.

**Fura 2 measures of [Ca<sup>2+</sup>]<sub>i</sub>.** Tracheal submucosal glands were isolated using a Wild zoom binocular dissecting microscope and transferred to 2 ml of HCO<sub>3</sub><sup>-</sup>-buffered Ringer solution (in mM: 125.0 NaCl, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 1.6 K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 1.0 MgCl<sub>2</sub>, 5.0 glucose, 10.0 Na acetate, 2.0 glycine, 1.0  $\alpha$ -ketoglutarate, 2.0 CaCl<sub>2</sub>, 25.0 NaHCO<sub>3</sub>) gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>. After 7 min of incubation in this buffer solution containing 2 mg/ml of collagenase NB 4 standard grade (SERVA) and 2 mg/ml of trypsin inhibitor (Sigma) at 37°C, submucosal glands were agitated gently using a fire-polished, wide-bore (1- to 2-mm) Pasteur pipette. After sedimentation, the gland cells were washed twice with enzyme-free bicarbonate-buffered Ringer solution. The isolated acinar fragments (consisting of clusters of up to 20 cells) were seeded onto glass coverslips (22  $\times$  22 mm) coated with poly-L-lysine and incubated for 30 min in physiological salt solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM HEPES, 10 mM glucose, and 5  $\mu$ M fura 2-AM (Teflabs) in the presence of Pluronic F-127 (Invitrogen) to enhance dye loading. Fura 2-loaded cells were mounted in a flow chamber on the stage of an inverted microscope (Nikon) for imaging; temperature was main-

tained at 37°C. The cells were illuminated at 340 and 380 nm, and the emitted fluorescence at 510 nm was collected with a charge-coupled device (CCD) camera and analyzed using the MetaFluor system (Universal Imaging). Fluorescence images were obtained at 2-s intervals; the fluorescence ratio [ratio of absorbance at 340 nm to absorbance at 380 nm (340/380)] was taken as the measure of [Ca<sup>2+</sup>]<sub>i</sub>.

**DIC imaging of isolated airway submucosal glands.** Tissues containing glands were isolated as described above and further microdissected to prepare relatively clean but intact glands for optimal imaging. When judged suitable for imaging, the glands were transferred to microperfusion chambers on the stage of an upright microscope (Eclipse E600FN Series, Nikon) equipped with DIC and epifluorescence. They were continuously perfused with Krebs solution gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> via a pressurized, eight-chamber, solenoid-actuated perfusion system (Automate). Temperature was maintained at 35–37°C using a TS-4 Peltier effect temperature controller that warmed the inflow tubing and chamber. For digital imaging, the microscope is fitted with a Retiga 1300, cooled, 12-bit, color Bayer Mosaic CCD camera with red-green-blue (RGB) liquid crystal color filter module. The camera is interfaced with a computer running Compix image capture-and-analysis software.

For overview images of the gland, we used a  $\times$ 4 objective. For detailed studies of cellular changes in the acini, tubules, and ducts of glands, glands were imaged with a  $\times$ 40 water immersion lens (0.8 numerical aperture, 2-mm working distance), and time-lapse digital imaging was used to monitor changes with frame rates of 2–30 s<sup>-1</sup>. At this magnification, the field and focal plane are sufficiently restricted, so that it is usually necessary to select a single duct, tubule, or acinus for optical imaging. These measures capture only a fraction of the dynamics of gland behavior during stimulation; therefore, video files have been provided as supplemental online material (see Supplemental Videos 1 and 2 in Supplemental Material for this article, available online at the Journal website).

Serous and mucous cells were distinguished by two criteria: location and physical appearance. Cells that were “bumpy” when viewed with DIC and were seen only in the acini of the gland were scored as serous cells; in these cells, the smaller apical surface usually appeared smaller than the basolateral surface. Cells in the tubules downstream from the acini appeared smooth when viewed with DIC and were scored as mucous cells; in contrast to the tapering serous cells, they were typically rectangular when viewed from the side. There was usually a clear demarcation between the two types of cells, sometimes indicated by a constriction. On occasion, we saw acini that appeared to be a continuation of the mucous tubule, with no discernible serous cells; these mucous acini have been described previously (26).

We used the following rationale for interpreting our data. A transection or longitudinal section through a duct, tubule, or acinus will appear as an annulus. The outer diameter (*R*) and the inner or luminal diameter (*r*) were measured frame-by-frame using Simple PCI running on a personal computer. Annular area =  $\pi(R^2 - r^2)$  is the area occupied by cells. The DIC method produces a thin optical slice through the acinus or tubule. Measurements were made only when two criteria were satisfied: 1) outer and inner edges of the annulus were clearly visible, and 2) the initial diameters were maximized (by through-focusing), so that we were measuring the center of the acinus/tubule. If *z*-axis drift occurred during stimulation, the data were not included in the analysis. A 20- $\mu$ m-thick section was assumed for conversion of all area measurements to volume. This assumption allowed comparison among tubules and acini and among different tissues. It was not possible to measure the volume of individual cells accurately, because all the cell's edges cannot be monitored simultaneously. Instead, we use the volume of the cell-containing annulus to monitor cell volume.

**Reagents.** Compounds were obtained from Sigma-Aldrich, Teflab, Invitrogen, Calbiochem, and SERVA. They were made fresh or maintained as stocks at -20°C. Stock solutions of SubP, phosphoramidon (a metalloproteinase inhibitor that helps prevent breakdown of

SubP by endopeptidase), carbachol, VIP, atropine (an inhibitor of muscarinic receptors), L-703606 (a NK-1 receptor blocker), and L-8-K (a VIP receptor blocker) were dissolved in sterile distilled water, and indomethacin (an inhibitor of prostaglandin release) was dissolved in ethanol. Stock solutions of forskolin, CFTR<sub>inh</sub>-172, niflumic acid (to inhibit CaCC channels), BAPTA-AM (to chelate [Ca<sup>2+</sup>]<sub>i</sub>), clotrimazole (to inhibit K<sub>Ca</sub> channels), MDL-12330A (to inhibit adenylate cyclase), and U-73122 (to inhibit PLC) were dissolved in DMSO and stored at -20°C. Bumetanide was dissolved in 0.1 M NaOH, and capsaicin was dissolved in mineral oil. All were diluted 1:1,000 with bath solution (except indomethacin, which was diluted 1:10,000) immediately before use at the concentrations indicated. The highest DMSO concentration in our experiments was 0.2%, and the highest ethanol concentration was 0.01%; our test of 0.5% DMSO as vehicle alone produced no effect.

**Statistics.** Values are means ± SE. Student's *t*-test for unpaired data or ANOVA was used to compare the means of different treatment groups, unless otherwise indicated. The difference between the two means was considered to be significant when *P* < 0.05. Curves were fit with Origin software (OriginLab) using a sigmoid function.

## RESULTS

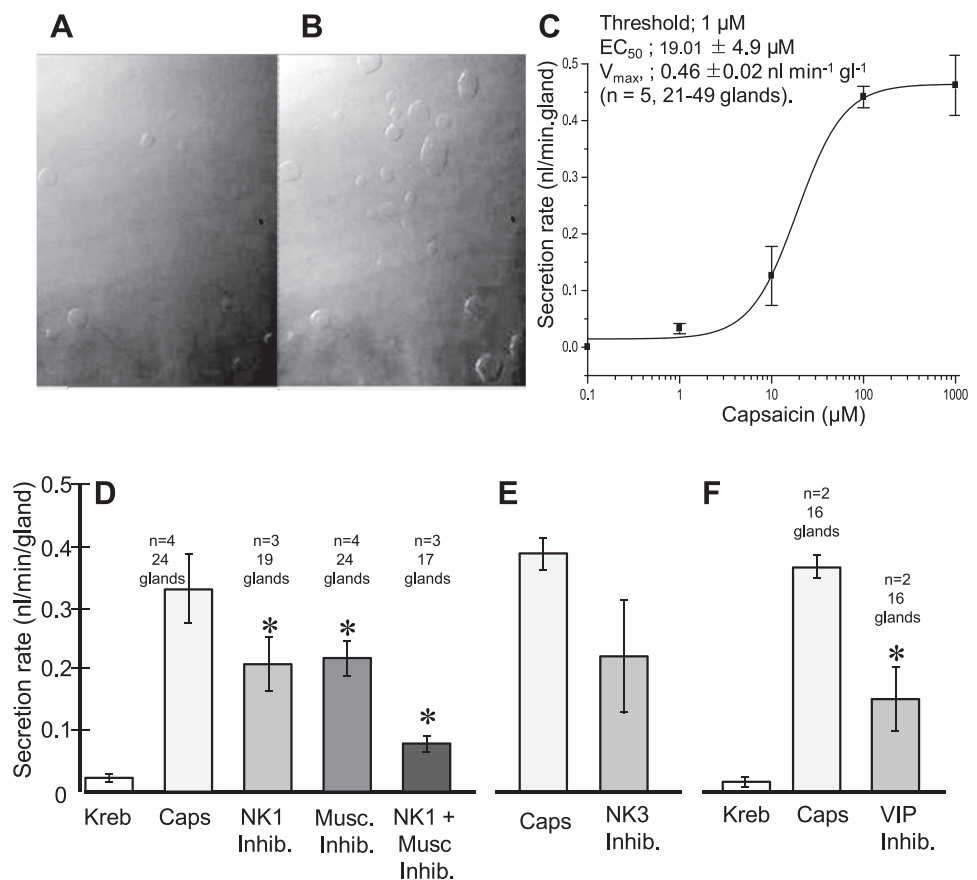
**Mucosal capsaicin weakly stimulates CFTR-dependent glandular mucus secretion.** Pig bronchial mucosa contain abundant SubP-containing C-fibers (21), which release SubP in response to capsaicin, and mouse and human tracheal submucosal glands secrete in response to mucosal chili oil via a CFTR-dependent mechanism (5, 14). We found that pig tracheal glands also secrete when capsaicin is added to the mineral oil coating the mucosa (Fig. 1, *A* and *B*). Mucosal capsaicin (≤100 μM) stimulated secretion in 30–40% of the glands that could

be stimulated with carbachol, with an approximate EC<sub>50</sub> of 19 ± 5 μM (Fig. 1*C*). Secretion rates were low in the responding glands. Stimulation is weak in these isolated mucosal preparations, presumably because all central reflexes are absent and only a fraction of the intrinsic neural circuitry innervating the glands (40) is intact. SubP-containing C-fibers can stimulate glands directly via axon reflex or indirectly via airway intrinsic neurons. In ferrets, airway intrinsic neurons that receive SubP innervation express SubP, VIP, and ACh (9). We used receptor antagonists to determine whether these transmitter systems participate in capsaicin-stimulated glandular secretion in pigs. Glandular fluid secretion to mucosal capsaicin was reduced 36–59% by antagonists against each of the three transmitter systems: the SubP receptors NK-1 and NK-3, VIP receptors, and muscarinic receptors (*n* = 21–39 glands from 3–6 pigs for each antagonist; Fig. 1, *D–F*).

We expected that capsaicin-induced secretion in pigs would be CFTR-dependent, as it is in mice and humans (5, 14). Because the number of CF pig tissues (33) was not sufficient to test this hypothesis directly, we incubated tissues with CFTR<sub>inh</sub>-172 (24) prior to stimulation. CFTR<sub>inh</sub>-172 reduced secretion rates by ~70% (*n* = 31 glands from 6 pigs, *P* < 0.05; Fig. 2*A*). Taken together, these results suggest that mucosal capsaicin stimulates glandular fluid secretion in isolated pig tracheas by releasing low levels of multiple mediators and that the response is at least partially CFTR-dependent (Fig. 2*B*).

**Serosal SubP strongly stimulates secretion from pig tracheal submucosal glands.** We next tested serosal application of SubP. Prior studies demonstrated that pig tracheal glands

Fig. 1. Mucosal capsaicin stimulated porcine submucosal gland secretion via multiple pathways. *A*: mucus bubbles basally secreted under the oil layer on the mucosal surface of a pig trachea over a 20-min period. *B*: tissue in *A* at 20 min after addition of 100 μM (calculated final concentration) capsaicin (Caps) to the oil layer. *C*: dose-response relation for gland secretion to mucosal capsaicin. Points represent mean secretion rate for glands that responded to 0.1, 1, 10, 100, and 1,000 μM capsaicin. Responding glands were defined as those that increased their secretion rate by >50% over basal secretion rate; basal rates were subtracted from secretion rates in the presence of capsaicin to give response rates. *D–F*: glandular secretion stimulated by mucosal capsaicin stimulation was partially blocked by inhibitors of substance P (SubP), VIP, and ACh (2–4 pigs and 16–24 glands for each condition). *D*: response to 100 μM mucosal capsaicin and partial inhibition by the neurokinin (NK-1) receptor blocker L-703606 (oxalate salt, 1 μM) and the muscarinic receptor blocker atropine (1 μM); their effects were additive. *E*: partial inhibition by the NK-3 receptor blocker SB-22200 (1 μM). *F*: partial inhibition by the VIP receptor inhibitor L-8-K (10 μM). \*Significantly different from capsaicin alone, *P* < 0.05. Krebs, Krebs-Henseleit buffer.





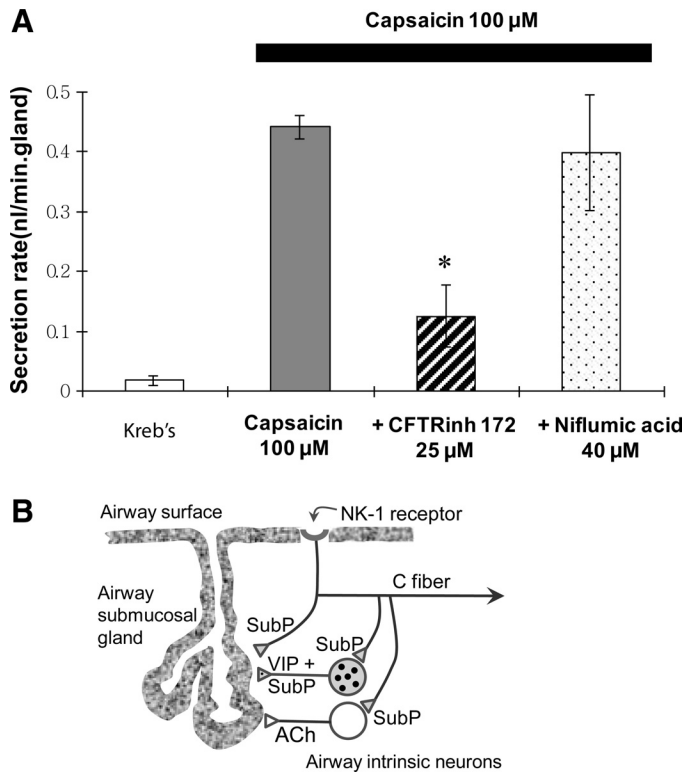


Fig. 2. Mucosal capsaicin in pig trachea stimulated CFTR-dependent glandular secretion. **A:** stimulation of submucosal gland secretion by mucosal capsaicin was partially inhibited by CFTR<sub>inh</sub>-172 (25  $\mu$ M), but not by niflumic acid (40  $\mu$ M). For each condition, 4–6 pigs and 17–31 glands were tested. \*Significantly different from capsaicin alone,  $P < 0.05$ . **B:** schematic summary diagram of innervation of porcine airway glands suggested by the data of Figs. 1 and 2A and anatomic evidence from pigs (21) and cats (8). Centrally originating C-fibers express NK-1 receptors on their processes in the airway mucosa and, via axon reflex, release SubP to stimulate airway glands directly and via intrinsic airway neurons, which express SubP, VIP (which can be in the same neurons; shaded area), and ACh.

secrete strongly in response to serosal SubP (1, 28, 37). We confirmed those results, and by measuring single-gland secretion rates at 1-min intervals, we demonstrated that the temporal time course is the same as that in response to carbachol (18), namely, a large, short-latency response that peaked in  $<5$  min and then quickly dropped to a rate of sustained secretion that was markedly less than the peak rate (Fig. 3A) and continued for as long as SubP was present ( $\leq 1$  h). This response pattern, which closely resembles the profile of  $[Ca^{2+}]_i$  observed in many cells stimulated with carbachol, including mouse nasal gland acinar cells (22, 23), differs markedly from the fluid secretory response to forskolin, which lacks the early peak (18). The secretion rates are  $\geq 10$  times greater to saturating doses of SubP than to mucosal capsaicin, and the responses have a different pharmacological profile from those mediated by capsaicin (see below). In Fig. 3B, approximate dose-response relations for SubP stimulation of pig and human airway glands are based on the amount of secretion observed in the first 15-min period following SubP application. As reported previously (5), SubP is much more efficacious in pigs than in humans; results in Fig. 3B show that it is also much more potent.

We compared the concentration-response relations of SubP with those of carbachol. In agreement with prior findings based

on stimulation of bulk mucus secretion from small bronchi (1, 37), in fresh pig tracheas, SubP and carbachol had similar efficacies for stimulation of mucus secretion from individual pig glands. Comparison of the dose-response curve to SubP with that of carbachol (4) reveals that SubP is also more potent than carbachol in producing glandular mucus secretions (Fig. 3C).

In addition to their similar response profiles, saturating doses of carbachol and SubP are nonadditive: the mean response averaged over 15 min to 10  $\mu$ M SubP was  $3.48 \pm 1.38$   $\text{nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$ , and this failed to increase upon addition of 10  $\mu$ M carbachol ( $3.21 \pm 1.05$   $\text{nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$ ,  $n = 23$  glands from 3 pigs,  $P > 0.05$ ). In contrast, when 10  $\mu$ M SubP

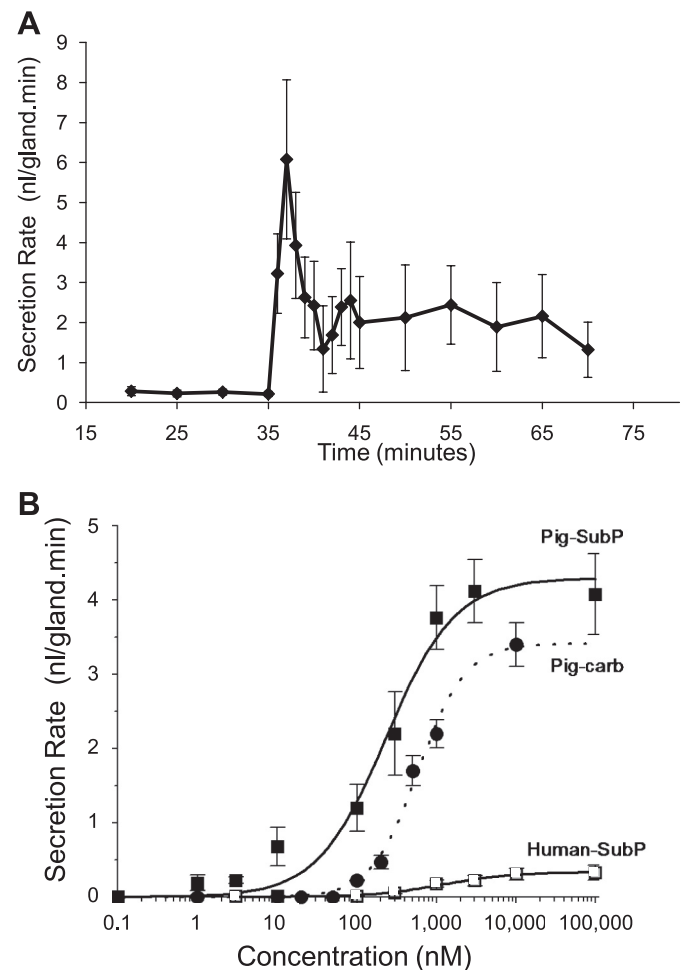


Fig. 3. Time course and dose-response relations of porcine glandular fluid secretion to SubP. **A:** time course of response to 10  $\mu$ M SubP and 1 mM phosphoramidon. Average secretion rates are shown for 18 glands from 3 pigs tested  $<4$  h after harvest. Rates were measured every minute for the initial 10 min and then every 5 min. **B:** dose-response relations for SubP-stimulated mucus secretion (solid lines) from pig (■) and human (□) and carbachol (Carb) dose-response for pig (dotted line, ●). For the pig SubP curve, each point represents 10–14 glands from each of 2–3 animals measured within 8 h of harvest. A maximum of 2–3 doses were checked sequentially after application of SubP, and their average secretion rates over 15 min were plotted;  $V_{\max} = 4.12 \pm 0.4$   $\text{nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$ , and  $EC_{50} = 230 \pm 60$  nM. For comparison with human SubP dose-response data, we replotted human data from Choi et al. (5);  $V_{\max} = 0.33 \pm 0.1$   $\text{nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$ , and  $EC_{50} = 1 \pm 0.2$   $\mu$ M. For comparison with carbachol-stimulated pig glandular secretion, we replotted carbachol dose-response data from Choi et al. (4).

was added to 1  $\mu\text{M}$  VIP, the mean secretion rate increased from  $0.79 \pm 0.47$  to  $2.91 \pm 1.57 \text{ nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$  ( $n = 32$  glands from 4 pigs,  $P < 0.05$ ).

**SubP-stimulated glandular mucus secretion is  $\text{Ca}^{2+}$ - and PLC-dependent.** SubP elevates  $[\text{Ca}^{2+}]_i$  in most systems where it has been studied, with a common signaling pathway as follows: SubP  $\rightarrow$  NK-1  $\rightarrow$   $\text{G}_q \rightarrow$  PLC  $\rightarrow$  inositol trisphosphate + diacylglycerol  $\rightarrow$   $\uparrow [\text{Ca}^{2+}]_i$  via inositol trisphosphate receptor- $\text{Ca}^{2+}$  channels in the endoplasmic reticulum and L-type plasma membrane  $\text{Ca}^{2+}$  channels (20). The contributions of extra- and intracellular  $\text{Ca}^{2+}$  and PLC were assessed by paired experiments in which secretion was stimulated in the presence of an inhibitor followed by stimulation of a paired tissue from the same pig without inhibitor. To determine whether increased  $[\text{Ca}^{2+}]_i$  is involved in SubP-stimulated glandular secretion, tissues were stimulated with 10  $\mu\text{M}$  SubP alone or after 30 min of incubation in 100  $\mu\text{M}$  BAPTA-AM, which should interfere with  $[\text{Ca}^{2+}]_i$  increases from intracellular stores and via influx. Secretion rates were decreased by 68% in BAPTA-AM (Fig. 4). Rates averaged over 20 min to SubP alone were  $2.23 \pm 0.57$  vs.  $0.73 \pm 0.13 \text{ nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$  for glands incubated in BAPTA-AM ( $n = 4$ , 28 bubbles,  $P < 0.05$ ). To determine whether extracellular  $\text{Ca}^{2+}$  was required, we stimulated glands with 1  $\mu\text{M}$  SubP in normal Krebs solution or in nominally  $\text{Ca}^{2+}$ -free Krebs solution (no added  $\text{Ca}^{2+}$ , 1 mM EGTA + 3 mM  $\text{Mg}^{2+}$ ). After 10–15 min of incubation in  $\text{Ca}^{2+}$ -free solution, gland responses averaged over 20 min were reduced by 90% compared with controls ( $0.21 \pm 0.08$  vs.  $2.17 \pm 0.65 \text{ nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$ ,  $n = 3$  pigs, 5–8 glands per pig,  $P < 0.05$ ; Fig. 4). To determine whether PLC was involved, SubP-stimulated secretion was measured in the presence of 50  $\mu\text{M}$  U-73122, a PLC inhibitor. This inhibited secretion by 60%, from  $2.4 \pm 0.65$  to  $0.95 \pm 0.19 \text{ nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$  ( $n = 4$  pigs, 6–8 glands per pig,  $P < 0.05$ ).

**Comparison of inhibitor effects on SubP- and carbachol-stimulated gland fluid secretion.** The mechanisms mediating gland secretion to SubP and carbachol were probed with various inhibitors. Pig tracheal glands were stimulated with SubP or carbachol in the presence of one of four inhibitors: 10  $\mu\text{M}$  atropine to inhibit muscarinic receptors, 25  $\mu\text{M}$  clotrimazole to inhibit  $\text{K}_{\text{Ca}}$  channels (7), 100  $\mu\text{M}$  MDL-12330A to

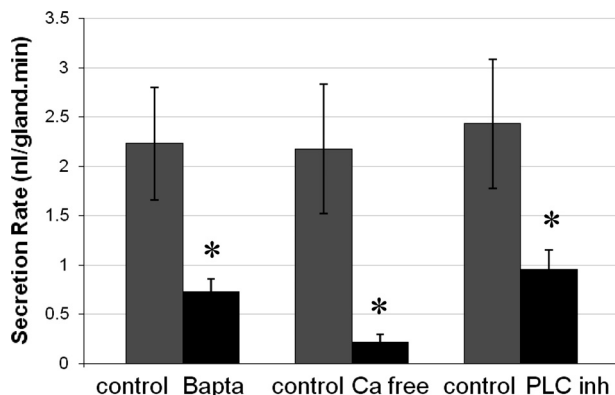


Fig. 4. SubP-stimulated gland secretion is  $\text{Ca}^{2+}$ -dependent. Pig submucosal glands were stimulated with 10  $\mu\text{M}$  SubP alone or in one of three conditions: 100  $\mu\text{M}$  BAPTA-AM (an intracellular  $\text{Ca}^{2+}$  chelator), a nominally  $\text{Ca}^{2+}$ -free bath solution (no added  $\text{Ca}^{2+}$ , 1 mM EGTA + 3  $\mu\text{M}$   $\text{Mg}^{2+}$ ), or 50  $\mu\text{M}$  U-73122 (a PLC inhibitor). Secretion was significantly reduced by each condition: \* $P < 0.05$ .

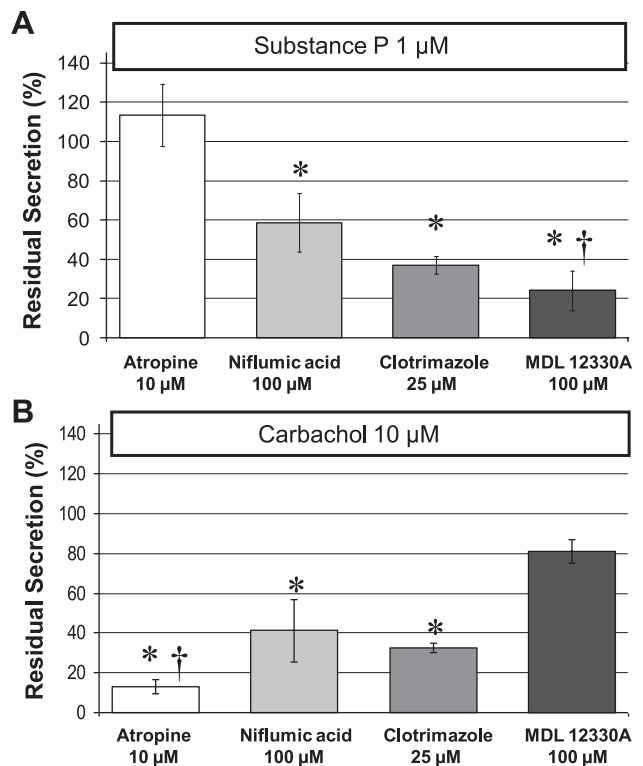


Fig. 5. Comparison of inhibitor effects on SubP- and carbachol-stimulated secretion. A: effects of atropine (to inhibit muscarinic receptors), niflumic acid (to inhibit  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels), clotrimazole (to inhibit  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels), and MDL-12330A (to inhibit adenylate cyclases) on SubP-stimulated secretion. Inhibitors were applied 20 min before agonist and were continuously present during the 20-min period after stimulation over which secretion was averaged. Each drug was tested on 29–41 glands from  $\geq 3$  pigs. Secretion to SubP alone was  $2.4 \pm 0.5 \text{ nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$ . B: effects of atropine, niflumic acid, clotrimazole, and MDL-12330A on carbachol-stimulated secretion. Secretion to carbachol alone was  $3.0 \pm 0.3 \text{ nl} \cdot \text{min}^{-1} \cdot \text{gland}^{-1}$ . \*Significantly different from SubP or carbachol alone,  $P < 0.05$ . †Significantly different from the other agonist tested with similar inhibitor  $P < 0.01$ .

inhibit adenylate cyclases (41), and 100  $\mu\text{M}$  niflumic acid, which, although not highly specific, inhibits  $\text{Ca}^{2+}$ -activated anion channels (38) more effectively than CFTR (4, 13, 14). Using separate pieces of tissue from the same pig, we compared the responses with responses to 1  $\mu\text{M}$  SubP or 10  $\mu\text{M}$  carbachol given alone or in the continuous presence of each inhibitor applied 10–20 min before the addition of agonist. Secretion rates were averaged for 20 min following agonist application. The inhibitor profiles for SubP and carbachol are shown in Fig. 5. As expected, atropine inhibited carbachol- but not SubP-stimulated secretion (37), while the inhibitors of  $\text{Ca}^{2+}$ -activated anion and  $\text{K}_{\text{Ca}}$  channels reduced secretion significantly ( $P < 0.05$ ) to each agonist.

The adenylate cyclase inhibitor MDL-12330A reduced SubP-mediated secretion by  $76 \pm 10\%$  ( $P < 0.01$ ) and that of carbachol by  $19 \pm 6\%$  [ $P = 0.26$  (not significant),  $P < 0.01$  for MDL inhibition of SubP vs. MDL inhibition of carbachol; Fig. 5]. To put these responses in perspective, we also determined the effect of MDL-12330A on glandular fluid secretion produced by 10  $\mu\text{M}$  forskolin. For the forskolin experiments, we compared secretion rates for 30 min following stimulation with and without inhibitor. The adenylate cyclase inhibitor reduced forskolin secretion by  $66 \pm 8\%$  ( $P < 0.02$ ,  $n = 26$  glands from

4 pigs). Thus, for the three agonists, the reduction in secretion by MDL-12330A can be ranked in the following order: SubP  $\approx$  forskolin  $>$  carbachol.

We also estimated changes in  $[Ca^{2+}]_i$  with fura 2 (Fig. 6). In unstimulated cells, the fluorescence ratio (340/380) was 0.32–0.36. A 90-s pulse of 10  $\mu$ M SubP increased the fluorescence ratio by 0.35–0.47 in 27 cells from 3 pigs (peak value). All 27 cells also responded to a 90-s pulse of 10  $\mu$ M carbachol with similar increases in fluorescence ratio of 0.37–0.42.

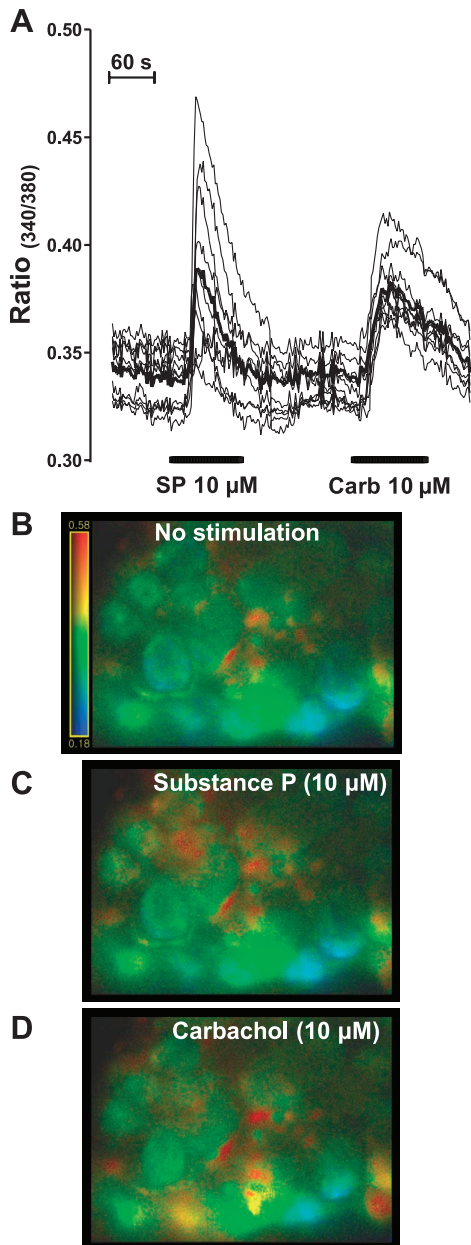


Fig. 6. Fura 2 responses of pig tracheal gland acinar cells to SubP and carbachol. Fura 2 ratio [ratio of fluorescence at 340 nm to fluorescence at 380 nm (340/380)] imaging confirms that SubP elevates cytosolic  $Ca^{2+}$ . A: results from a representative experiment showing 1- to 2-s pulses of 10  $\mu$ M SubP (SP) and carbachol (Carb) caused similar elevations of  $Ca^{2+}$  concentration in partially dissociated acinar cells of pig submucosal glands (10–20 acinar cells were usually still associated). Each trace corresponds to measures from a single cell. B–D: representative images for peak responses in A; color range is 340/380 = 0.18–0.58.

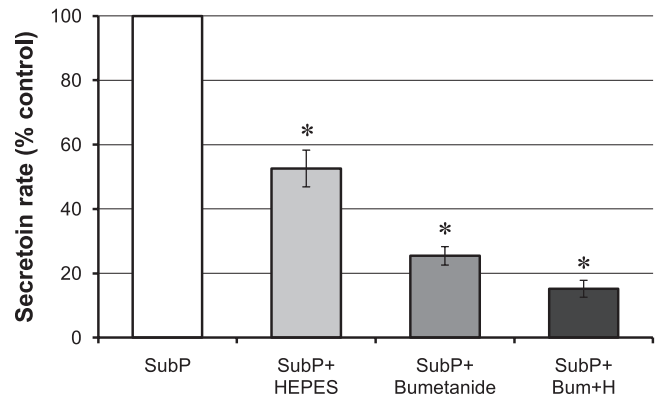


Fig. 7. Anion dependence of SubP-stimulated glandular fluid secretion. Open bar represents mean control secretion rate to 10  $\mu$ M SubP measured in Krebs-Ringer bicarbonate solution of  $2.6 \pm 0.4$  nl·min<sup>-1</sup>·gland<sup>-1</sup> set to 100%; subsequent bars show mean residual percentage of control secretion rate in the presence of HEPES, bumetanide (Bum), and HEPES + bumetanide. Each treatment produced a significant decrease in secretion vs. control: \* $P < 0.05$ . Each condition represents mean data from 25–30 glands in each of 5 pigs.

**Anion dependence of SubP-stimulated glandular fluid secretion.** SubP stimulation of bulk mucus secretion from pig bronchial segments is anion-dependent (37). We measured mucus secretion from individual glands and assessed its anion dependence by inhibiting  $Cl^-$  transport with bumetanide and  $HCO_3^-$  transport with HEPES replacement of  $HCO_3^-$  and gassing with  $O_2$ . For each of five animals, we determined the secretion rate in response to SubP alone and to each of the inhibitor conditions. To average the effects of the ion transport inhibition, we normalized the secretion rates by setting the control rate to 100 for each pig and then expressing the rates after anion transport inhibitions as a percentage of the control rate (Fig. 7). Secretion was decreased to  $52.6 \pm 5.7\%$  of control by HEPES replacement, to  $25.4 \pm 2.9\%$  by bumetanide, and to  $15.2 \pm 2.6\%$  by HEPES + bumetanide (mean  $\pm$  SE,  $n = 5$ ,  $P < 0.01$  for all conditions relative to control). These results confirm the anion dependence of SubP-stimulated glandular fluid secretion.

**Responses to SubP decline rapidly over time after tissue is harvested.** Carbachol-stimulated glandular mucus secretion is relatively stable over 48 h when the tissue is stored at 4°C prior to testing (18). In contrast, secretion rates and the number of glands responding to SubP declined significantly with time after harvest. When fitted with a single exponential, the secretion rate decayed with a half time of 16 h (Fig. 8A). We did not independently measure secretion rates to carbachol alone in this series of experiments. However, we followed SubP stimulation with carbachol stimulation and measured the proportion of glands secreting to SubP vs. carbachol as a function of time after harvest. Here we also saw a significant ( $P < 0.01$ ) decline in SubP responsiveness relative to carbachol responsiveness (Fig. 8B).

**Gland cell responses to SubP studied with DIC microscopy.** The effects of SubP on individual cells within glands were observed with DIC optics using time-lapse digital imaging while SubP (1–10  $\mu$ M) was superfused over isolated pig glands ( $n = 6$  pigs). Responses displayed the following features: 1) rapid cell shrinkage of serous and mucous cells by 15–60% of their volumes; 2) increased flow of mucus from the serous acini and along tubules and ducts; 3) increased exocytosis;



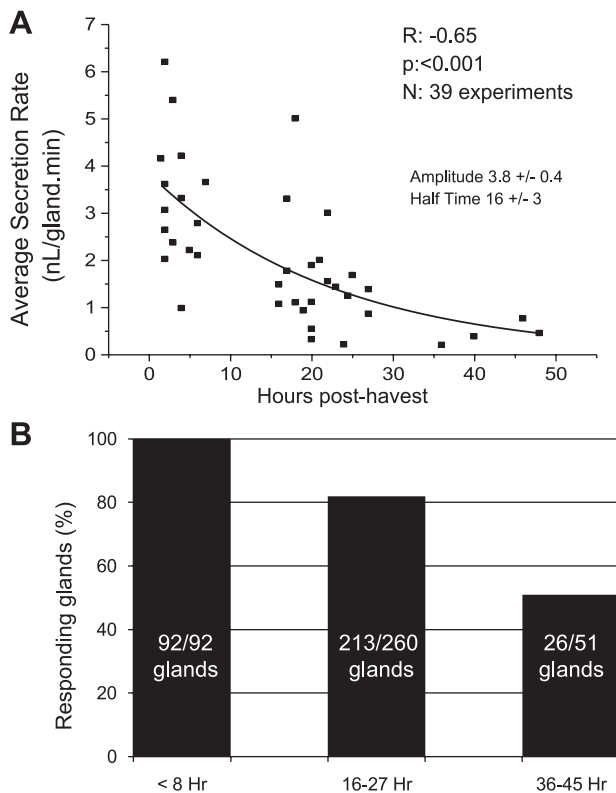


Fig. 8. Decline in glandular secretion to SubP as a function of time after tissue harvest. **A**: mean secretory rate of 6–14 glands to 1  $\mu$ M SubP in a single experiment. Regression analysis showed that secretion to SubP declined significantly as a function of time after harvest, with a half time of 16 h ( $P < 0.01$ ,  $n = 39$  experiments). **B**: percentage of carbachol-responsive glands (10  $\mu$ M) that responded to 1  $\mu$ M SubP as a function of time after harvest. Number of glands that were tested and responded during each time period is shown within bars; 5–9 pigs were tested at each time period.

4) absence of myoepithelial cell contractions in most glands and small, transient contractions in some; and 5) persistence of these effects in the presence of 1  $\mu$ M atropine (which abolished the response to 1  $\mu$ M carbachol; data not shown). In contrast with SubP, 1  $\mu$ M carbachol caused powerful contractions of myoepithelial cells (Figs. 9 and 10; also see Supplemental Videos 1 and 2).

## DISCUSSION

Chronic airway infections are the main health burden on people with CF, placing a premium on understanding how they start. The innate defenses of the upper airways include antimicrobial-rich mucus produced by submucosal glands, which express CFTR. The glands secrete mucus in response to multiple inputs that differentially engage CFTR-dependent and -independent fluid secretion, and we hypothesize that defects in CFTR-mediated gland secretion contribute to the impaired ability of human CF airways to eradicate bacteria.

CF pigs provide an unprecedented opportunity to study the development of CF lung disease, because they, like humans with CF, are born with airways that have defective ion transport in their surface epithelia, defective innate defenses against bacterial infection, and defective tracheal gland secretion (16, 33, 36). CF pigs go on to develop airway disease with many features similar to humans with CF (36). Pig lungs share many

features with human lungs (31), but they are not identical, and as the analysis of lung disease in CF pigs proceeds, there is increased need to compare the details of structure and function of pig and human lungs.

Prior studies in mice and humans of SubP-stimulated glandular fluid secretion showed that secretion was primarily CFTR-dependent and was scant compared with cholinergically stimulated secretion (5, 13, 14). However, Martens and Ballard (25) and Trout et al. (37) demonstrated that SubP powerfully stimulated secretion of fluid,  $\text{Cl}^-$ , and  $\text{HCO}_3^-$  into the bronchial lumen of pig airways without affecting absorption; they were able to attribute secretion to glands, rather than surface epithelia, by removing the surface epithelia in some experiments without consequence for secretion. Therefore, it seemed important to quantify SubP-stimulated mucus secretion in pigs using the same methods we used for mice and humans.

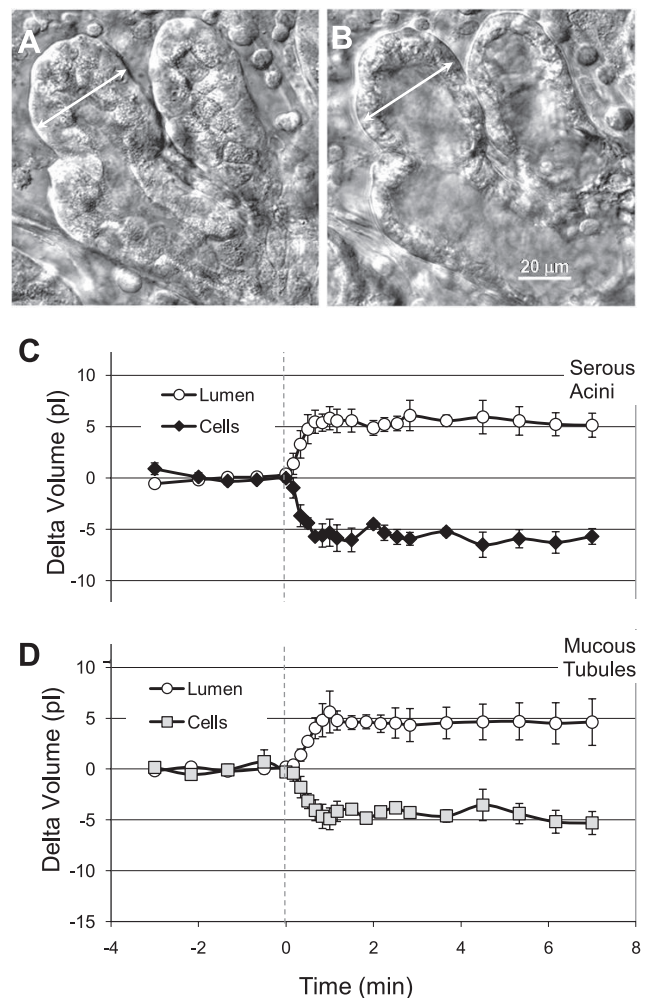


Fig. 9. SubP stimulates cell shrinkage and mucus flow but little or no myoepithelial cell contraction. **A** and **B**: differential interference contrast (DIC) monitoring of cell and lumen volume changes in pig submucosal glands stimulated with 1  $\mu$ M SubP. **A**: 3 serous acini and 2 mucous tubules prior to stimulation. **B**: acini in **A** at peak response to superfusion of 1  $\mu$ M SubP. External diameter of acini (arrows) does not diminish, as it does with carbachol, but, instead, increases slightly as a result of hydrostatic pressure produced by secretion into the lumen. **C** and **D**: mean volume changes as a function of time and stimulation with 1  $\mu$ M SubP for the acinar lumen and serous cells (serous acini) and corresponding increases in mucous tubule lumen and decreases in gland mucous cells ( $n = 3$ ).

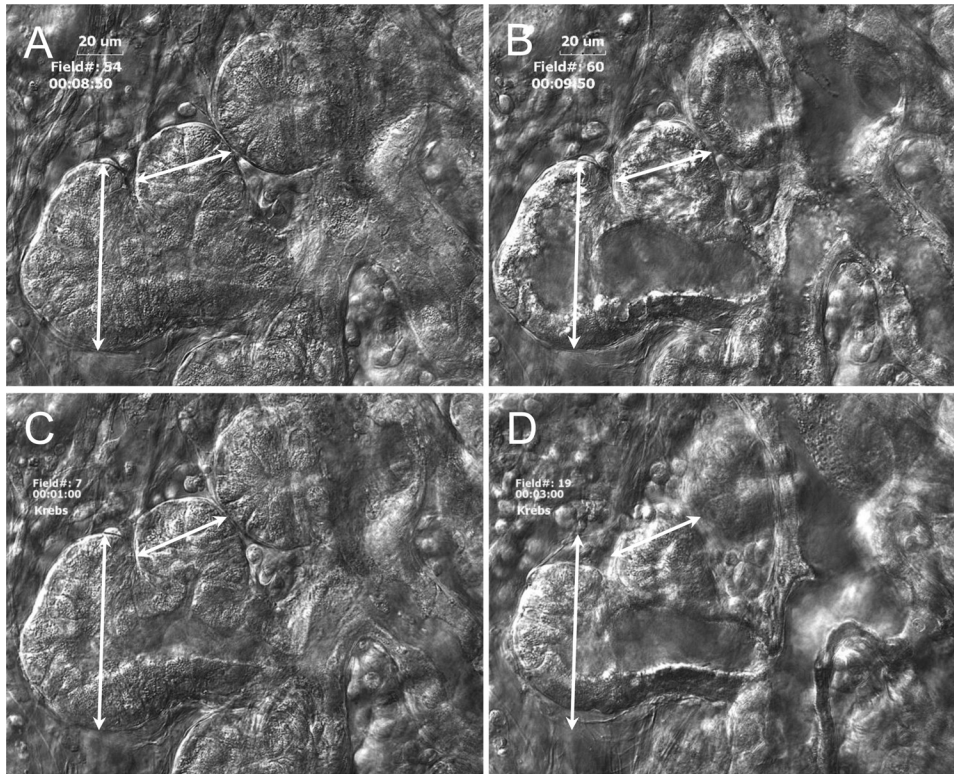


Fig. 10. SubP and carbachol stimulation of pig tracheal submucosal glands. *A* and *B*: DIC monitoring of cell and lumen volume changes in a different pig from Fig. 9. *A*: serous acini and associated tubules prior to stimulation. Arrows show extent of acini at rest; the same arrows were transposed on the image in *B*, which shows the same acini 30 s after the first visible response to superfusion of 10  $\mu$ M SubP, which consisted of massive cell shrinkage and lumen expansion and occurred  $\sim$ 50 s after the start of SubP flow. *C* and *D*: acini in *A* and *B* before and after stimulation with a 1-min pulse of 1  $\mu$ M carbachol. Carbachol flow started at 1 min and stopped at 2 min. The first response, at  $\sim$ 40 s, consisted of myoepithelial contraction and was followed,  $\sim$ 30 s later, by cell shrinkage.

In the present study, we found that submucosal glands in isolated pig tracheal mucosa modeled human glands by responding weakly to mucosal capsaicin (5). The axon reflex pathway used multiple transmitters, and the response was at least partly dependent on CFTR. In contrast with the weak responses to mucosal stimulation, serosal application of SubP produced copious, anion-dependent secretion. For pig tracheal glands, serosal SubP was as efficacious as carbachol and considerably more potent. This stands in contrast with human tracheal glands, where SubP is much less potent and effica-

cious than carbachol (5). Thus, pig and human tracheal glands differ profoundly in their responsiveness to SubP. The gland secretory responses to SubP, mucosal capsaicinoids, and carbachol are compared for control and CF mice, humans, and pigs in Table 1.

Four lines of evidence indicate that the tracheal gland fluid secretory response to SubP in pigs was mediated at least in part by elevated  $[Ca^{2+}]_i$ . 1) The secretory response rate over time showed a sharp transient peak very similar to that seen with the  $Ca^{2+}$ -elevating agonist carbachol (Fig. 3A). 2) Secretory re-

Table 1. Secretory responses to SubP, capsaicinoids, and carbachol in mice, humans, and pigs

	Mouse		Human		Pig		
	WT	CF	WT	CF	Adult WT	Neonatal Control	Neonatal CF
<i>Tracheal/bronchial gland</i>							
Mucosal capsaicin or chili oil	0.05 <sup>a</sup>	0 <sup>a</sup>	0.3 <sup>c</sup>	0 <sup>c</sup>	0.4 <sup>d</sup>	ND <sup>e</sup>	ND <sup>e</sup>
Serosal SubP	0.1 <sup>a</sup>	0 <sup>a</sup>	0.25 <sup>c</sup>	0 <sup>c</sup>	2.5 <sup>d</sup>	0.15 <sup>e</sup>	0.06 <sup>e</sup>
Carbachol	1.4 <sup>b</sup>	1.4 <sup>b</sup>	2.5 <sup>c</sup>	2.5 <sup>c</sup>	2.0 <sup>c</sup>	0.4 <sup>e</sup>	0.3 <sup>e</sup>
<i>Nasal gland</i>							
Pilocarpine			$\sim$ 12 <sup>f</sup>	$\sim$ 4.5 <sup>f</sup>			
<i>Normalized to carbachol in WT</i>							
Mucosal capsaicin	3.5	0	12	0	20	ND	ND
Serosal SubP	7.1	0	10	0	125	37.5	15
Carbachol	100	100	100	100	100	100	75
Pilocarpine			100	37			

Values are expressed as  $nl \cdot min^{-1} \cdot gland^{-1}$ . Tracheal/bronchial gland secretory rates for mouse are mainly peak values (first 5 min); others are averages of peak and sustained values (first 15 min). Substance P (SubP) was applied at 1  $\mu$ M and carbachol at 10  $\mu$ M, except in neonatal pigs, where carbachol was 1  $\mu$ M. Different normalized results of human control and CF glands stimulated with carbachol or pilocarpine are thought to represent the different states of the glands tested (hypertrophied in CF or not), rather than the agonist used. CF, cystic fibrosis; WT, wild-type; ND, not determined. <sup>a</sup>Ianowski et al. (13); <sup>b</sup>Ianowski et al. (14); <sup>c</sup>Choi et al. (4); <sup>d</sup>present study; <sup>e</sup>Joo et al. (16); <sup>f</sup>Salinas et al. (34).



sponses were significantly reduced by BAPTA-AM, nominally  $\text{Ca}^{2+}$ -free Ringer solution, or inhibition of PLC (Fig. 4). 3) Secretory responses were significantly reduced by inhibitors of  $\text{Ca}^{2+}$ -activated anion or  $\text{K}_{\text{Ca}}$  channels (Fig. 5). 4) Fura 2 fluorescence ratio imaging of dissociated gland acinar cells showed that SubP increased  $[\text{Ca}^{2+}]_i$  in a fashion similar to carbachol (Fig. 6).

We confirmed the finding that atropine does not inhibit the gland secretory response to SubP (37), indicating that none of these responses are mediated by a secondary release of ACh (Fig. 5). Although similar overall, SubP-stimulated glandular fluid secretion differed in three ways from carbachol-stimulated secretion. 1) It was significantly more sensitive to the adenylate cyclase inhibitor MDL-12330A (Fig. 5), suggesting involvement of CFTR. 2) It declined more rapidly as a function of time after tissue harvest (Fig. 8), a feature also shared by forskolin-stimulated secretion. 3) DIC imaging of the gland acini revealed that SubP caused virtually no contraction of the myoepithelial cells surrounding the glands, in contrast with powerful contractions observed with carbachol (Figs. 9 and 10). {It is interesting that copious fluid secretion can be produced without the need for myoepithelial cell contractions; it is also clear that the early spike in the secretion rate (Fig. 3A) is not the result of myoepithelial contraction but, instead, probably reflects a spike in  $[\text{Ca}^{2+}]_i$  that is seen with many  $[\text{Ca}^{2+}]_i$ -elevating agonists. However, it is unlikely that these glands would ever be stimulated purely by SubP under physiological conditions.}

The much greater potency and efficacy of SubP for stimulating pig vs. human tracheal glands are important because of evidence that high levels of  $[\text{Ca}^{2+}]_i$  recruit CaCCs, whereas lower levels, while still sufficient to activate basolateral  $\text{K}_{\text{Ca}}$  channels, do not recruit CaCCs (4, 5). The significance of this is that tracheal gland secretion in response to SubP in humans appears to be mainly CFTR-dependent (16). However, in pigs, we predict that SubP will produce a higher level of  $[\text{Ca}^{2+}]_i$ , which will activate CaCCs and, thus, render secretion at least partly CFTR-independent. Limited experiments with neonatal control and CF piglets provide support for this expectation (16). The work in neonatal piglets was limited by the small amounts of tissue available, but enough was done to be confident that SubP-stimulated tracheal gland secretion is already robust at birth (16) and that it persists in CF piglets, although it is significantly reduced in volume (Table 1).

Single-gland, fluid volume studies of tracheal gland mucus secretion stimulated by mucosal capsaicin or serosal SubP have been carried out in control and CF humans, control and CF mice, adult pigs (this study), and control and CF neonatal piglets (Table 1). As shown in Table 1, in mice and humans, the responses to serosal SubP are <10% of the responses to carbachol, and these smaller responses seem to depend almost entirely on CFTR. However, responses to SubP in pigs are much larger and partly independent of CFTR. Because we do not understand the role of glands or of SubP in airway innate defenses, the consequences of the differences for CF lung disease in the two species are unknown.

In these experiments, central reflexes were absent and local axon reflexes were degraded by the removal of many local neurons during dissection. It is unknown if the residual secretory responses to strong mucosal stimulation are simply scaled down versions of full responses or if their properties have

changed. The latter seems likely on the basis of the evidence that increasing levels of  $[\text{Ca}^{2+}]_i$  recruit CaCCs and make the response less CFTR-dependent.

We quantified the volume of mucus secreted over time, but we did not measure features such as pH, viscosity, or the levels and types of mucins and antimicrobials present. These properties are impossible to measure when CF glands are stimulated by pathways that elevate intracellular cAMP concentration, because no mucus bubbles can be observed with this method over 20–30 min of stimulation in CF mice, piglets, or humans (Table 1). However, in mucus produced by CF glands in response to cholinergic stimulation, pH is lower and viscosity is higher than normal, suggesting that CFTR and CaCCs play a role in fluid formation (15, 17, 34, 35). Alterations in  $\text{HCO}_3^-$  secretion may be particularly important for mucus formation (11, 27, 29, 30). Thus it will be important to measure these properties in control and CF pig tracheal glands stimulated with SubP and carbachol. SubP and carbachol might produce mucus with different properties, even when they produce similar volumes; for example, cultured submucosal gland cells from sheep secreted almost five times more lysozyme in response to 10  $\mu\text{M}$  SubP than to 20  $\mu\text{M}$  methacholine (12), although the volume of mucus secreted in response to SubP has not been studied in sheep glands.

These experiments have focused on tracheal glands, but it will also be informative to compare nasal glands in pigs and humans. In the human nasal cavity, SubP is responsible for most of the unilateral effects following instillation of hypertonic saline, including pain, rhinorrhea, and increased lavage recovery of total protein, lactoferrin, mucoglycoprotein markers, and SubP itself, with only minor reflex-mediated effects in the contralateral nasal cavity (3). These are attributed to direct effects of SubP on NK-1 receptors on submucosal glands, while vascular processes, including plasma exudation and mucosal edema, which are prominent responses to SubP in the rodent nasal cavity, were not observed in humans (3). These results, which contrast with our studies of human tracheal glands (5), suggest a more important role for SubP in the nose than in the trachea and bronchi of humans.

In summary, studies of airway submucosal glands are revealing regional and species differences in responsiveness that presumably reflect differences in innervation. It will be important to determine how these differences affect the ability of glands to contribute to airway mucosal innate defenses in the absence of CFTR.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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